The EMBO Journal vol.7 no.8 pp.2547 – 2553, 1988

Active site mutagenesis of the AIDS virus protease and its alleviation by *trans* complementation

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Communicated by M.L.Birnstiel

Replacement of the putative active site Asp residue of cloned HIV-1 protease with Ala yields a molecule incapable of autocatalytic processing. Similarly, protease/reverse transcriptase and protease/reverse transcriptase/endonuclease polyproteins containing the same mutation accumulate as enzymatically inert polyproteins. Introduction of a second, wild-type, copy of protease *in trans* alleviates this defect, leading in the case of individually cloned protease to cleavage of the mutant protein, and with the polyprotein mutants to release of the reverse transcriptase and endonuclease polypeptides, the former of which recover enzymatic activity. In related experiments, a similar inhibition and *trans*-complementation of a genetically engineered *gag*–protease fusion protein was observed.

Key words: aspartyl proteinase/human immunodeficiency virus/polyprotein processing/reverse transcriptase

Introduction

The human immunodeficiency virus, type 1 (HIV-1) *pol* gene encodes a 115-kd polyprotein comprising a protease (mol. wt 17 kd), reverse transcriptase (mol. wt 64 kd) and endonuclease (mol. wt 34 kd) (Ratner *et al.*, 1985; Lightfoote *et al.*, 1986). Through presumably the *pol*-encoded protease, this polyprotein is processed to the respective components, wherein reverse transcriptase appears as polypeptides of mol. wt 64 and 52 kd (Di Marzo Veronese *et al.*, 1986). Cloning all or part of the HIV-1 *pol* gene in eukaryotic (Barr *et al.*, 1987) or bacterial systems (Tanese *et al.*, 1986; Farmerie *et al.*, 1987; Hansen *et al.*, 1987; Larder *et al.*, 1987; Le Grice *et al.*, 1987; Mous *et al.*, 1988) results in similar processing and accumulation of enzymatically active reverse transcriptase. These systems thus offer an attractive means of studying protease-mediated processing, especially in understanding the protease recognition sequence.

We have recently demonstrated in an *Escherichia coli* expression system that release of protease from the polyprotein is a two-step event whereby the full-length molecule undergoes autocatalytic processing to polypeptides of 7 and 10 kd (Mous *et al.*, 1988). Furthermore, it has recently been shown that biological activity resides in the 10-kd form (Debouck *et al.*, 1988; Graves *et al.*, 1988). Our discovery of a related 10-kd protease in virus preparations (S.F.J.Le Grice and J.Mous, unpublished) suggests this 10-kd polypeptide as the active protease form. Comparison of amino acid sequences of HIV-1 protease and other retroviral proteases reveals a common heptapeptide, -Leu-Leu-Asp-Thr-Gly-Ala-Asp-, within which the tripeptide -Asp-Thr-Gly- implicates it as an aspartyl proteinase (Katoh *et al.*, 1987; Pearl and Taylor, 1987). Such classification would imply that alteration of the active site Asp residue of this tripeptide would interrupt protease-mediated processing of the HIV-1 *pol* precursor polyprotein.

In this communication, we describe the effect of mutation of the presumptive active site Asp residue on processing of cloned protease, *pol* polyprotein forms and a genetically engineered *gag*–protease fusion protein. Whilst wild-type protease undergoes rapid autocatalysis to the 10-kd form, mutant protease accumulates as a full-length molecule. *Pol* polyproteins and the *gag*-protease fusion proteins containing the protease mutation likewise accumulate as precursor, wherein the former display no reverse transcriptase activity. Adding an independent transcriptional unit containing a copy of the wild-type protease gene to the respective plasmids restores the processing programme of individually cloned protease and mutant polyproteins, resulting in the relevant cases to reappearance of reverse transcriptase activity. These results unambiguously demonstrate that, in heterologous expression systems, the biologically significant cleavage events on the *pol* polyprotein, as well as those releasing p24 from the *gag* precursor, are mediated through the *pol*-encoded protease. Furthermore, it would appear that no unique cleavage recognition sequence exists for protease within these polyproteins.

Results

The Asp–Ala mutation abolishes internal protease cleavage

We and others (Debouck *et al.*, 1988; Graves *et al.*, 1988; Mous *et al.*, 1988) recently demonstrated in *E.coli* that HIV-1 protease is released from the *pol* polyprotein and immediately cleaved to a labile amino-terminal and stable

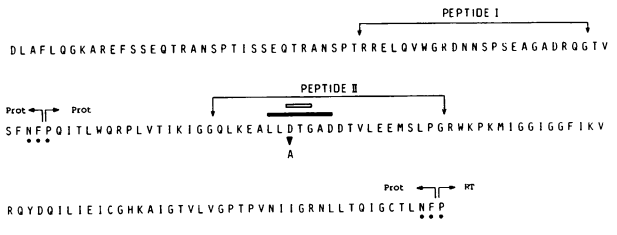

Fig. 1. Amino acid sequence of the HIV-1 protease in our bacterial expression system. Sequences at the internal protease cleavage site as well as those delineating protease from reverse transcriptase are indicated (●). Amino acids within the closed bar represent a sequence preserved between retroviral proteases. Within this, the open bar represents the homology between retroviral proteases and aspartyl proteinases (Katoh *et al.*, 1987; Pearl and Taylor, 1987). Peptide sequences against which antibodies were raised have been illustrated. Amino acid residues are symbolized by the letter code.

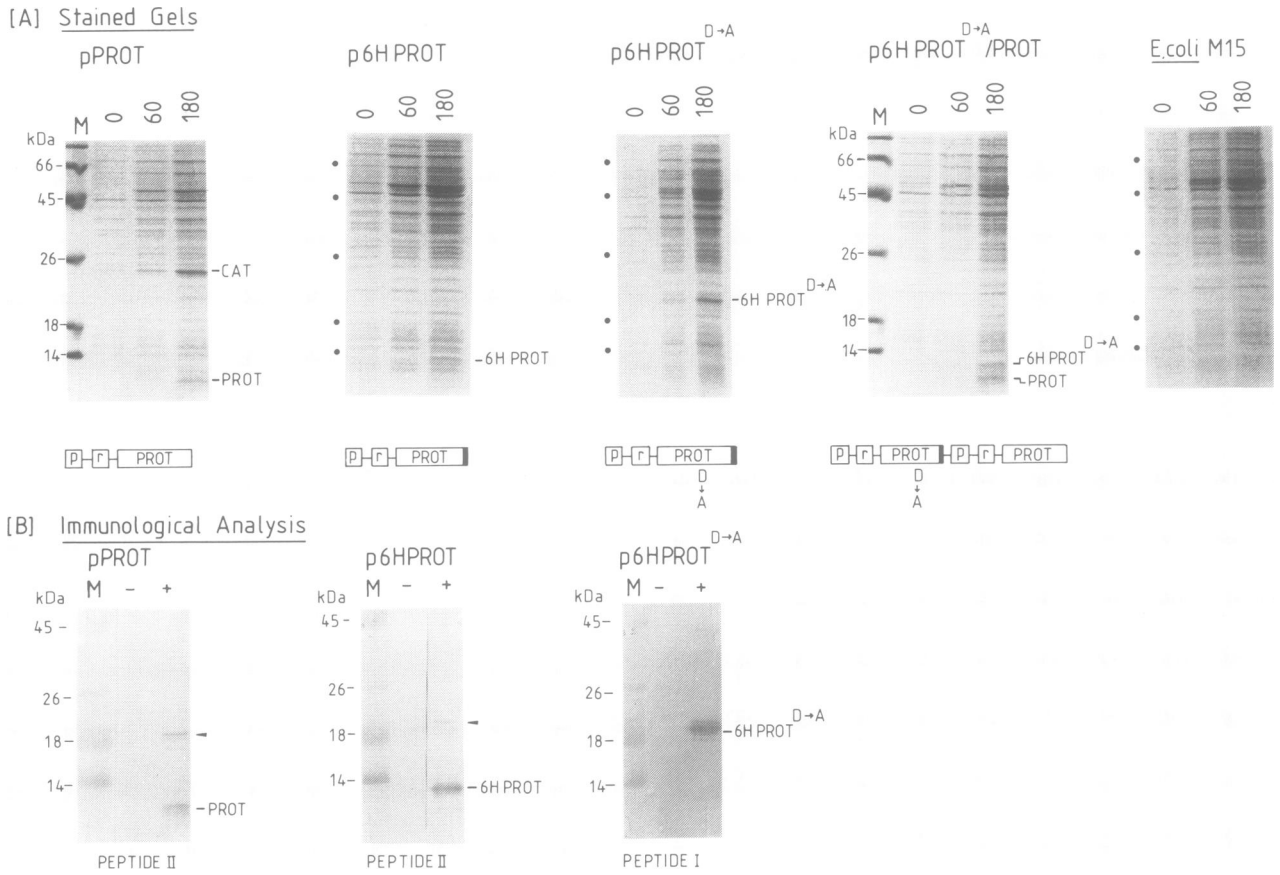

Fig. 2. Cleavage properties of wild-type (pPROT, p6HPROT), mutant (p6HPROT$^{D\rightarrow A}$) and *trans*-complemented (p6HPROT$^{D\rightarrow A}$/PROT) HIV-1 protease in *E.coli*, following IPTG induction. Presented are stained SDS−PAGE gels (**upper**) and an immunological analysis (**lower**). The *E.coli* strain harbouring pPROT also produces a 25-kd form of chloramphenicol acetyl transferase (CAT), as indicated on the stained gel. The nature of the antibodies used in the immunological assay is presented below each panel, as is the transcriptional unit present on the relevant plasmid. Within these transcriptional units, p refers to a synthetic T5 promoter/*lac* operator element (Stüber *et al.*, 1984), and r to a synthetic ribosome binding site. Numbers above each panel represent the post-IPTG induction time (min) at which aliquots of the cultures were removed for analysis. The filled box at the end of certain protease coding sequences represents a hexahistidine label, used to differentiate between wild-type and *trans*-complemented species.

carboxy-terminal portion, outlined diagrammatically in Figure 1. In an initial experiment, we determined the effect of an Asp−Ala mutation in the presumptive active site of the cloned protease moiety. Recombinant clones expressing full-length protease (pPROT), or a version extended at its carboxy terminus by six histidine residues (p6HPROT) direct accumulation of the carboxy-terminal portion (Figure 2A), illustrating the autocatalytic property of HIV-1 protease. In contrast, expression of the carboxy-terminal extended protease harbouring the Asp−Ala mutation (p6HPROT$^{D\rightarrow A}$) results in accumulation of full-length precursor. In the immunological analysis presented in Figure 2B, mutant protease from plasmid p6HPROT$^{D\rightarrow A}$ could only be detected with antibodies raised against protease Peptide I, since a consequence of the Asp−Ala mutation was destruction of the single, continuous epitope recognized by Peptide II antibodies (data not shown). From the results in Figure 2, we further postulate that internal cleavage of HIV-1 protease is not mediated via an *E.coli* protease.

Alternatively, alteration of the Asp residue could result in production of a protein which had lost its conformational integrity rather than enzymatic activity. Hence, we cloned a second copy of wild-type, full-length protease as a separate transcriptional unit into the plasmid p6HPROT$^{D\rightarrow A}$. As seen in Figure 2, the resulting strain (p6HPROT$^{D\rightarrow A}$/PROT) no longer accumulates the full-length molecule. For the reason stated above, immunological detection of the cleavage products of both wild-type and mutant protease was not possible. However, visualization of the *trans*-complementation process was possible by making use of the slightly larger molecular mass of the protease species extended at their carboxy terminus by histidine residues. In the stained gel analysis in Figure 2, we show that two carboxy-terminal portions of slightly different molecular mass accumulate in the strain p6HPROT$^{D\rightarrow A}$/PROT, demonstrating that mutant protease has retained a conformation accessible to the *trans*-complementing wild-type molecule.

Analysis of the Asp−Ala mutation in the context of a genetically engineered gag−protease fusion protein

Since the major function of HIV-1 protease is cleavage of the 55-kd *gag* precursor, we determined the effect of the protease mutation on a polypeptide wherein the *gag*- and protease-coding sequences were fused in-frame. In cells harbouring the plasmid pGAG-PROT, a 24-kd polypeptide, corresponding to *gag* p24, was released from the 70-kd precursor fusion polypeptide (Figure 3), illustrating protease-mediated cleavage of the *gag* precursor. Detection of the

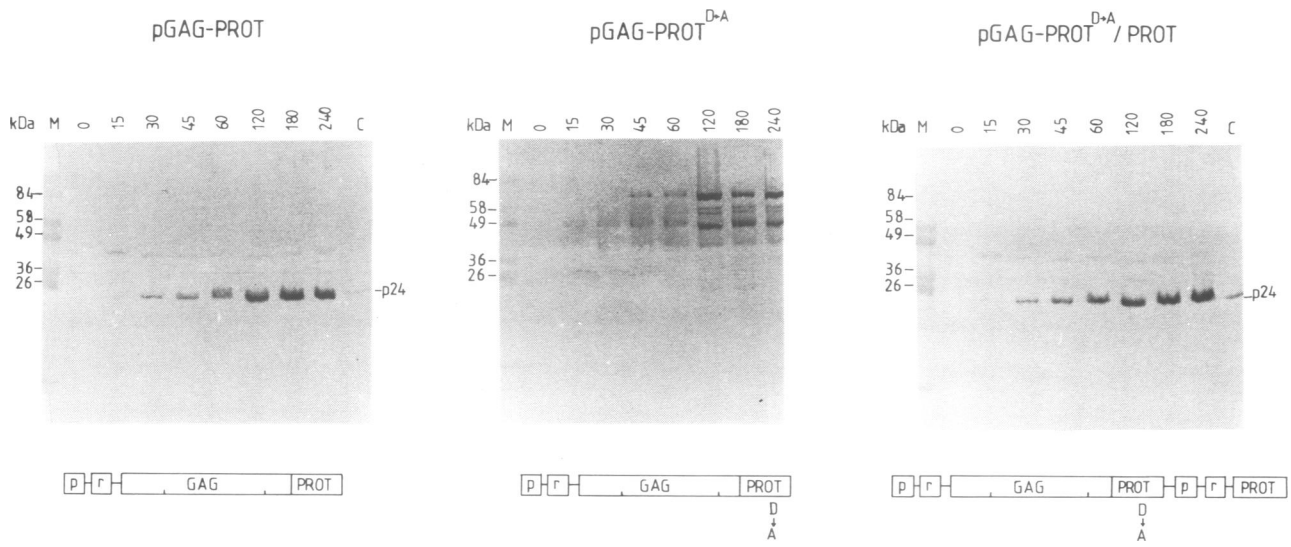

Fig. 3. Processing of wild-type (pGAG-PROT), mutant (pGAG-PROT$^{D\rightarrow A}$) and *trans*-complemented (pGAG-PROT$^{D\rightarrow A}$/PROT forms of a *gag*−protease fusion polypeptide. The immunoblot assay presented was accomplished using a p24-specific monoclonal Ab. The post-IPTG induction times (min) are indicated above each panel, the nature of the transcriptional units below each panel. The control lane (C) is a sample of partially purified HIV antigens, M represents pre-stained protein markers. The position of the HIV core protein p24 is indicated.

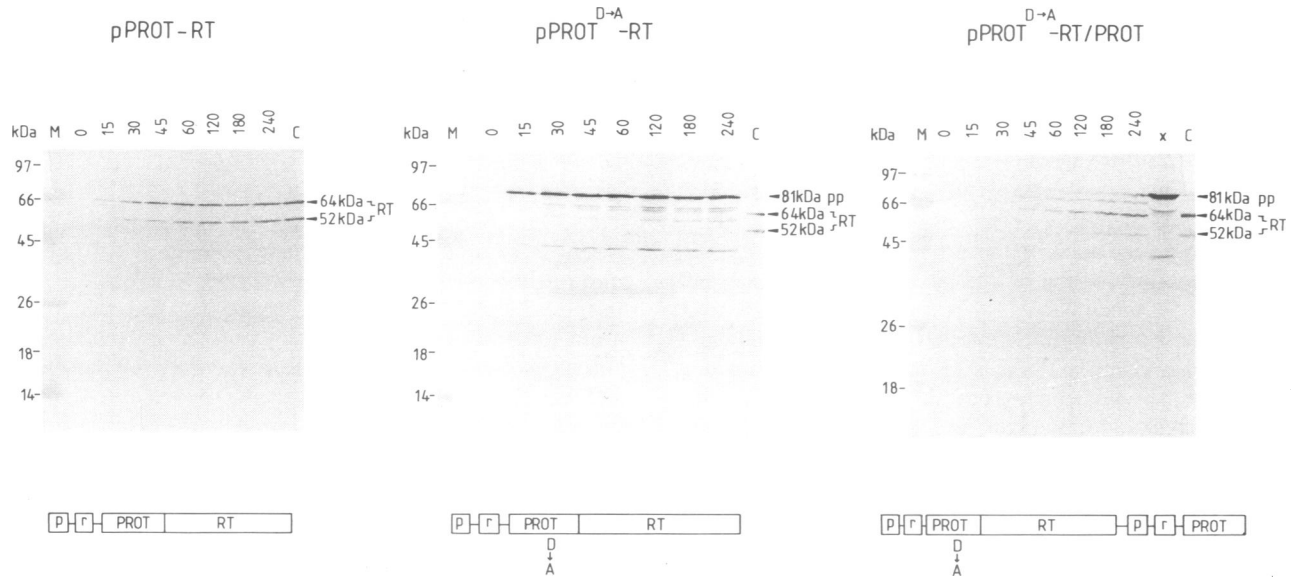

Fig. 4. Cleavage properties of wild-type (pPROT-RT), mutant (pPROT$^{D\rightarrow A}$-RT) and *trans*-complemented (pPROT$^{D\rightarrow A}$-RT/PROT) forms of an HIV-1 protease/reverse transcriptase polyprotein. An immunological analysis has been presented, wherein immunoreactive polypeptides were analysed using a pool of HIV-1-positive sera. Post-IPTG induction times (min) are indicated above, and the nature of the transcriptional unit in the relevant plasmid below each panel. The position of the 81-kd protease/reverse transcriptase polyprotein (pp) has been indicated on the panel pPROT$^{D\rightarrow A}$-RT. Control lane (C) is a sample of purified HIV-1 reverse transcriptase. Molecular weights were determined from a sample of pre-stained protein markers (**lane M**).

10-kd protease form with Peptide II antibodies (data not shown) furthermore indicated that protease was released from the fusion molecule. The same figure shows that introduction of the protease mutation (pGAG-PROT$^{D\rightarrow A}$) yields the full-length fusion polypeptide and some breakdown products (which are often observed when heterologous proteins are expressed in *E.coli*), but no polypeptide corresponding in size to p24. Restoration of the processing programme and reappearance of p24 was achieved when a wild-type copy of the protease gene was introduced in the same plasmid (pGAG-PROT$^{D\rightarrow A}$/PROT).

Cleavage properties of pol polyproteins containing mutated protease

Trans-complementation of the protease mutation made possible a study of *pol* processing in the context of this mutation. The importance of this study was to determine (i) whether all *pol*-processing events were mediated through its own protease and not a cellular protease, and (ii) if appearance of reverse transcriptase activity required that one or both of the respective polypeptides be matured from the precursor polyprotein. As a consequence, we genetically engineered the *pol* open reading frame (ORF) to produce

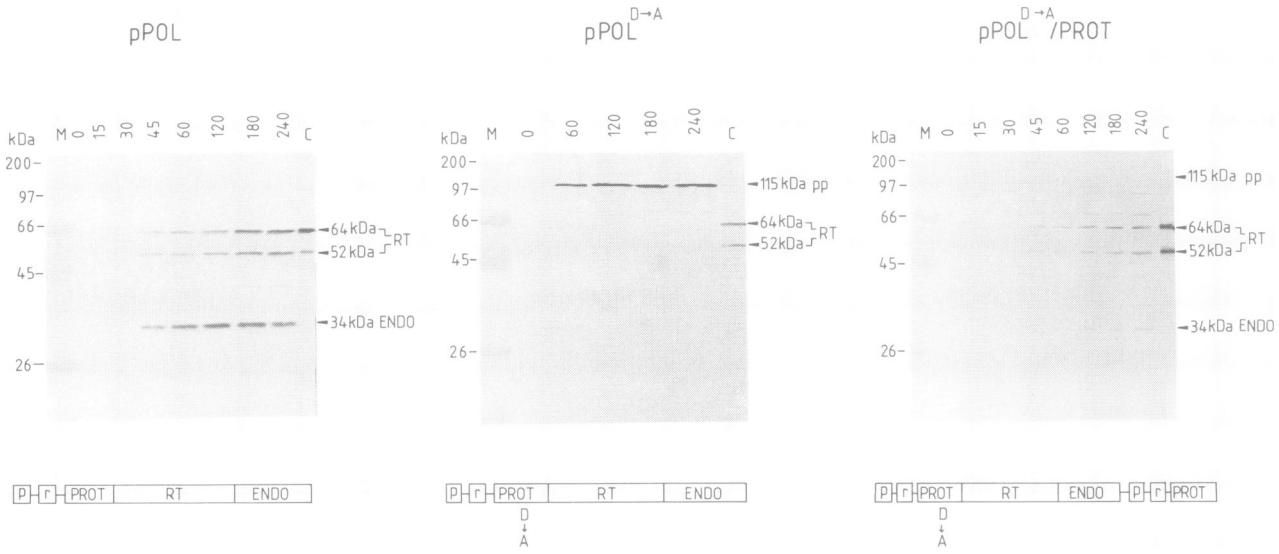

Fig. 5. Processing of wild-type (pPOL), mutant (pPOL$^{D\rightarrow A}$) and *trans*-complemented (pPOL$^{D\rightarrow A}$/PROT) forms of the entire HIV-1 *pol* precursor polyprotein. The immunological assay presented was again accomplished using a pool of HIV-1-positive sera. Notations above and below each panel are as described in the legend to Figure 3. The protein migrating at 34 kd in the strain harbouring pPOL and pPOL$^{D\rightarrow A}$/PROT was designated endonuclease (ENDO) from its cross-reactivity with antibodies raised against a peptide from the endonuclease coding sequence (data not shown). In the panel pPOL$^{D\rightarrow A}$, the position of the 115-kd, full-length precursor polyprotein (pp) is indicated. Control lane (C) is again a sample of purified HIV-1 reverse transcriptase.

a polyprotein lacking the endonuclease moiety (pPROT-RT). Based on the amino-terminal amino acid analysis of HIV-1 endonuclease (Lightfoote *et al.*, 1986), we introduced a stop codon following the presumptive last reverse transcriptase residue via a synthetic DNA adaptor. By doing so, we excluded the possibility that our results were influenced by unnecessary amino acids at the carboxy terminus of reverse transcriptase. Expression of this polyprotein from plasmid pPROT-RT is accompanied by processing to the 64- and 52-kd forms of reverse transcriptase (Figure 4). An immunological analysis with antibodies to protease Peptides I and II indicated simultaneous processing of protease to the carboxy-terminal form (data not shown). In contrast, polyprotein harbouring the Asp−Ala mutation in protease (pPROT$^{D\rightarrow A}$-RT) accumulates as an 81-kd protease/reverse transcriptase precursor. Although breakdown products are observed, these do not correspond to the size of reverse transcriptase. This non-specific breakdown was confirmed in reverse transcriptase assays (see later), where no enzymatic activity was observed. Supplying a copy of the protease sequence on the same plasmid (pPROT$^{D\rightarrow A}$-RT/PROT) restores the processing programme to yield the same products as from pPROT-RT. Finally, Figure 5 illustrates that the processing programme of the entire *pol* precursor protein expressed from plasmid pPOL is likewise completely abolished when the protease moiety contains the active site mutation (pPOL$^{D\rightarrow A}$), generating a 115-kd full-length *pol* polyprotein, and restored when a second copy of protease coding sequence was introduced into the same plasmid (pPOL$^{D\rightarrow A}$/PROT).

Restoration of the processing programmes of polyproteins from plasmids pPROT$^{D\rightarrow A}$-RT and pPOL$^{D\rightarrow A}$ has wider consequences other than demonstrating that the *pol*-encoded protease is responsible for all cleavage events. In this context, it is interesting to note that the amino acid sequence -Asn-Phe-Pro-, present at the internal protease cleavage site as well as the cleavage site delineating protease from reverse transcriptase, exists neither at the reverse transcriptase/endonuclease junction, nor at a position in reverse transcriptase coding sequence which would specify production of a 52-kd polypeptide.

Analysis of reverse transcriptase activity in wild-type, mutant and trans-complemented polyproteins

In the previous section we illustrated how introduction of a mutation into HIV-1 protease results in accumulation of various *pol* polyprotein forms. Restoration of the processing pathway demonstrates that these polyproteins have the conformational integrity to be recognized by the complementing copy of protease. We subsequently determined whether reverse transcriptase activity existed in these polyproteins, or whether it was associated only with the 64- and 52-kd reverse transcriptase polypeptides following their maturation from polyprotein.

In Figure 6 we present reverse transcriptase activity profiles and immunological analyses with partially purified preparations from the various protease/reverse transcriptase (Figure 6A) or protease/reverse transcriptase/endonuclease polyproteins (Figure 6B). From these results, it is clear that insignificant reverse transcriptase activity is detected in the protease/reverse transcriptase or full-length *pol* polyprotein. Extremely low level activity is associated with the protease/reverse transcriptase polyprotein, although we cannot rule out the possibility here of a low frequency processing which escapes the detection limits of our immunological analysis. As expected, significant levels of reverse transcriptase activity were recovered from the strains harbouring pPROT$^{D\rightarrow A}$-RT/PROT and pPOL$^{D\rightarrow A}$/PROT. In both cases, this level reached 40% of the wild-type counterpart: interestingly, the data in Figures 4 and 5 indicate that addition of a second copy of protease does not lead to full processing of the mutant polyproteins. We suspect that this is

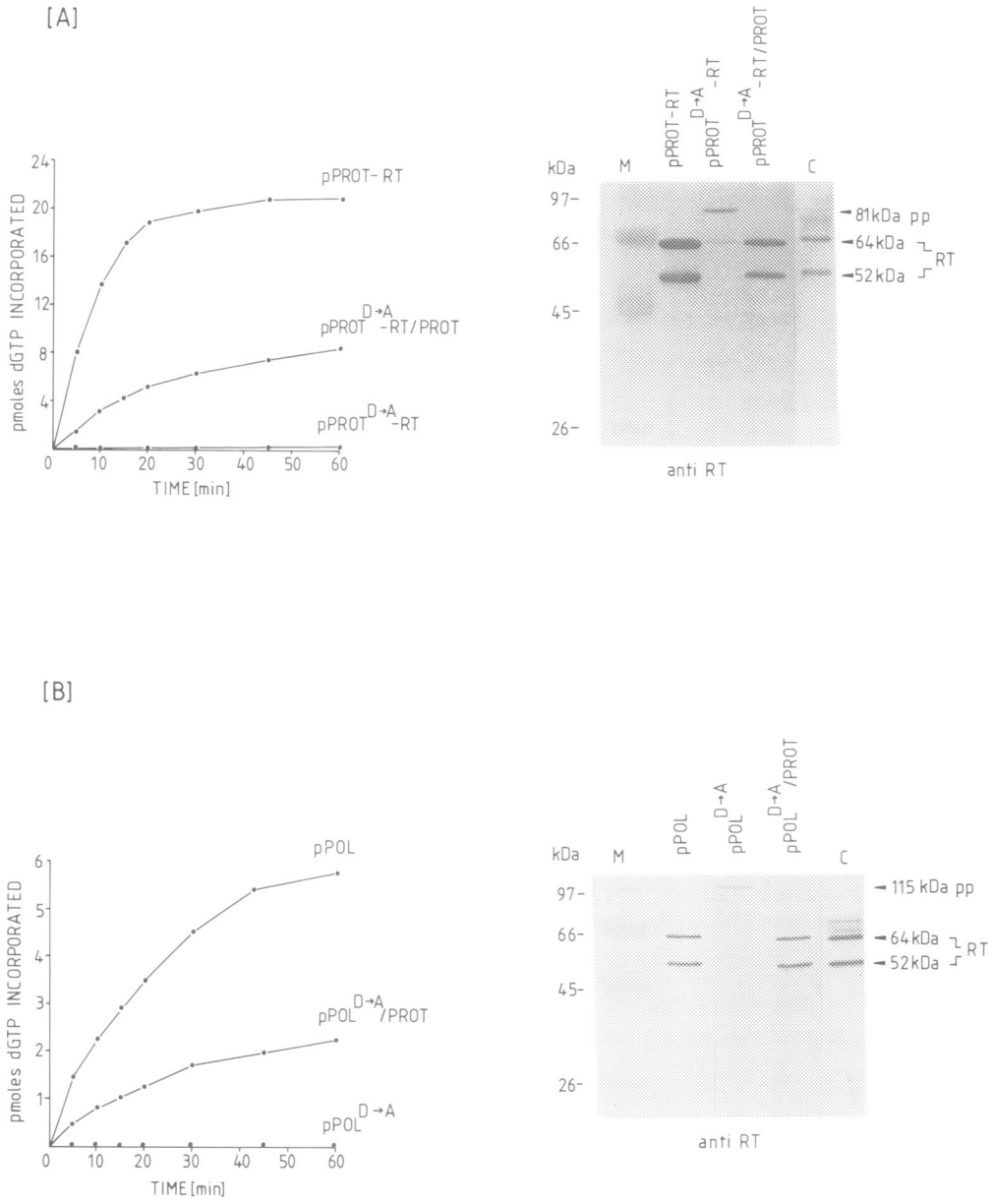

Fig. 6. (**A**) Reverse transcriptase activity profile (**left**) and immunological analysis (**right**) of protease/reverse transcriptase polyprotein forms. For uniformity, enzyme preparations from all three strains were performed in parallel. The activity profile presented was performed on 1:20 diluted, DEAE-Sephacel-purified enzymes. (**B**) Activity profile (**left**) and immunological analysis (**right**) of *pol* polyprotein forms. Isolation and assay was identical to that in (A). Note that the lower level of incorporation in series (B) is due to lower levels of production in the relevant *E.coli* strain rather than variations in enzyme activity. In both immunological analyses, polyclonal antibodies against purified HIV-1 reverse transcriptase were used. The control lane (C) is a sample of bacterially produced reverse transcriptase. Note that the rabbit antiserum used reacts with some contaminating *E.coli* proteins.

a consequence of a certain proportion of polyprotein forming insoluble aggregates. Finally, to ensure that the inactive polyproteins had indeed been isolated, we analysed all extracts following DEAE-Sephacel purification for immunoreactive polypeptides using antibodies directed against purified HIV-1 reverse transcriptase. From Figure 6, it is clear that correctly sized, unprocessed polyproteins could indeed be isolated by our protocol.

Discussion

Cloning and expression of the HIV-1 *pol* polyprotein in heterologous systems have been reported by several groups (Tanese *et al.*, 1986; Farmerie *et al.*, 1987; Hansen *et al.*, 1987; Larder *et al.*, 1987; Le Grice *et al.*, 1987; Mous *et al.*, 1988), monitored by the appearance of reverse transcriptase polypeptides together with enzymatic activity. However, it remains to be fully elucidated whether processing of the full-length polyprotein is mediated fully by the *pol*-specified protease, or whether a cellular protease co-participates. Similarly, it has not yet been fully established whether appearance of reverse transcriptase activity is a consequence of its maturation from the polyprotein, or whether processing intermediates have activity. In a previous report (Mous *et al.*, 1988), we showed that in *E.coli*, the ultimate product of the *pol* protease moiety is a 10-kd species

cleaved from a 17-kd precursor. Recent reports (Debouck *et al.*, 1988, Graves *et al.*, 1988) have furthermore indicated that proteolytic activity is associated with this 10-kd product. Mutational analysis of protease would thus answer certain of the questions we have posed.

The postulation that HIV-1 protease belongs to the family of aspartyl proteinases (Katoh *et al.*, 1987; Pearl and Taylor, 1987) suggested that the aspartate residue within the conserved -Asp-Thr-Gly- sequence was an obvious candidate for mutational studies. An Asp−Ala mutation was chosen to minimize unfavourable steric contacts and to avoid imposing new charge interactions or hydrogen bonds (Carter and Wells, 1988). We show here that a direct consequence of this mutation in cloned protease is accumulation of an enzymatically inert full-length molecule, in contrast to wild-type protease which can still cleave itself to the 10-kd form. Restoration of protease processing by *trans*-complementation illustrates that mutant protease retains a conformation acceptable for recognition by its wild-type counterpart. These results do not, however, exclude the possibility that the full-length form of protease is active. Such would be possible by mutating the internal cleavage junction of protease without destroying the active site. Experiments of this nature are presently underway.

A second feature of our results is that two forms of the *pol* polyprotein harbouring the active site protease mutation accumulate as the intact 81- or 115-kd precursor. Restoration of the processing pathway of these polypeptides indicates that all cleavage events are indeed HIV-1 protease mediated. In total, four processing events occur on the *pol* precursor polyprotein: (i) internal cleavage of protease, (ii) cleavage of protease from reverse transcriptase, (iii) internal cleavage of reverse transcriptase to release the 52-kd form and (iv) cleavage between reverse transcriptase and endonuclease to liberate the 64- and 34-kd polypeptides respectively. It is perhaps not surprising to find that all cleavages are mediated via the *pol* protease; however, what is interesting is the amino acid sequence at the respective junctions. The sequence -Asn-Phe-Pro- occurs at the junction between protease and reverse transcriptase, as well as within protease at a position which would correspond to production of the 7- and 10-kd protease forms we have earlier detected. Amino acid sequence analysis (Di Marzo Veronese, 1986; Lightfoote *et al.*, 1986) furthermore has established proline as the first residue of reverse transcriptase; this implies that the aforementioned tripeptide sequence is a good candidate for the protease recognition sequence. This or a related sequence is not found at the reverse transcriptase/endonuclease junction or within the reverse transcriptase coding sequence which would specify the 52-kd form. These results imply that no absolute protease recognition sequence exists, but rather primary and secondary cleavage junctions. When the *pol* gene product is considered in the context of the *gag−pol* fusion protein, the sequence -Asn-Tyr-Pro- is found at the junction between the *gag* moiety p17 and p24 (Ratner *et al.*, 1985). This or a related sequence is again absent at positions which would delineate reverse transcriptase from endonuclease or at the relevant position within reverse transcriptase. Such would strongly suggest that further mutational analysis be undertaken to alter amino acids at relevant cleavage junctions to determine their role in protease recognition.

Finally, we show here that semi-purified polyprotein forms display little or no reverse transcriptase activity, from which we propose that appearance of activity requires maturation of one or both of the reverse transcriptase polypeptides from the precursor polyprotein. Our results with the cloned HIV-1 *pol* gene confirm a previous observation of Witte and Baltimore (1978), who found that reverse transcriptase of the Moloney Murine leukaemia virus was enzymatically active only after cleavage from the 180 kd *gag−pol* polyprotein, which occurs after virions are released from cells. This was suggested as a mechanism for preventing premature initiation of reverse transcriptase before infection.

In a related study, Debouk *et al.* (1987) have shown that a *gag*-protease fusion protein modified via either amino acid insertion or premature termination within protease is likewise incapable of autolytic cleavage. However, these authors have not addressed the possibility that a consequence of alteration is production of an insoluble protein, indicating the importance of the *trans*-complementation experiments presented here.

Materials and methods

Microbiological manipulations

All recombinant DNA work was with the *E.coli* strain M15 harbouring the *lac* repressor-producing plasmid pDMI.1 (Certa *et al.*, 1986). Our expression system has been described (Certa *et al.*, 1986) and is under the transcriptional control of a synthetic T5 promoter/*lac* operator element. As HIV-1 *pol* gene, we used a *BglII−NdeI* fragment of the HTLV IIIB provirus (Ratner *et al.*, 1985). The *gag* gene used to generate an in-frame *gag*−protease fusion protein was an *XmnI−BglII* fragment of the same provirus. Rather than indicating the nature of each expression vector, we have presented with our results the relevant transcriptional unit(s) within these vectors.

For isopropyl-β-D-thiogalactopyranoside (IPTG) induction experiments, recombinant strains were grown in antibiotic-supplemented L-broth to OD_{600} = 0.7, after which IPTG was added to a final concentration of 400 μg/ml. At times indicated in the text, portions were removed and processed for analysis via either stained protein gels or immunoreactive polypeptides. Small-scale reverse transcriptase purifications were made from 500-ml, 5-h-IPTG induced cultures.

Electrophoretic and immunological analysis

One-millilitre portions of induced cultures were collected by centrifugation, resuspended in 200 μl of SDS−PAGE sample buffer (Laemmli, 1970), and heated for 10 min at 90°C. Aliquots (10 μl) were fractionated through either 10 or 12.5% SDS−polyacrylamide gels containing a 3.3% stacking gel (Laemmli, 1970). Fractionated proteins were either stained with Coomassie Brilliant Blue (Serva), or electrophoretically transferred to nitrocellulose according to Towbin *et al.* (1979). Immunological detection was accomplished with the following antibody preparations: (i) a pool of thermally inactivated HIV-1$^+$ serum, (ii) rabbit polyclonal antibodies to a peptide spanning amino acids 39−62 of HIV-1 protease (Peptide I), (iii) rabbit polyclonal antibodies to a peptide spanning amino acids 86−108 of HIV-1 protease (Peptide II, see Figure 1), (iv) rabbit polyclonal antibodies to purified HIV-1 reverse transcriptase and (v), a monoclonal antibody directed against HIV-1 *gag* p24. Colorimetric determination of immunoreactive polypeptides with HIV-1 serum was with horseradish peroxidase-coupled second antibody as previously described (Certa *et al.*, 1986). In all other cases, colour development was via an alkaline phosphatase assay system from Bio-Rad. For reference, a prestained marker mix, containing proteins in the mol. wt range 14−200 kd (Gibco-BRL) was run in parallel.

Purification and assay of HIV-1 reverse transcriptase

Conditions for small-scale reverse transcriptase purification via DEAE-Sephacel ion-exchange chromatography have been described (Le Grice *et al.*, 1987). Under our conditions, reverse transcriptase was recovered as the non-DEAE-Sephacel binding fraction. All enzymatic assays were performed with such preparations. Assays reported here were in 150 μl, in a solution of 50 mM Tris−HCl, pH 8.0, 50 mM NaCl, 5 mM dithiothreitol, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100, 10 μg/ml poly(rC), 5 μg/ml oligo(dG), 5 μM dGTP and 0.5 μCi [α-^{32}P]dGTP (800 Ci/mmol) and aliquots of

reverse transcriptase as indicated. At times indicated in the text, 7.5-μl portions were spotted onto DEAE paper filters. Once dry, filters were washed three times in 2 × SSC, (SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.6), twice in ethanol and again dried. Radioactive incorporation was determined in 2 ml of scintillation fluid.

In vitro site-directed mutagenesis

Site-directed mutagenesis of the protease-active site Asp residue to Ala, as well as modification of the *pol* polyprotein at the RT/ENDO junction, was accomplished by the method of Taylor *et al.* (1985). To delineate reverse transcriptase from endonuclease, we initially mutated the DNA sequence -AAA-ATA-CTA- at this junction (Ratner *et al.*, 1985) to -AAA-ATA-TTA-, thus introducing as a silent mutation an *Ssp*I site (AATATT). In a similar fashion, the *Ssp*I site within the reverse transcriptase coding sequence was eliminated as a silent mutation. The integrity of these mutations was confirmed by DNA sequencing, using the dideoxy chain termination method of Sanger and Coulson (1977).

Routine materials

Restriction enzymes were from Biolabs or Pharmacia, DEAE Sephacel from Pharmacia and radionuclides from Amersham. Routine chemicals were from Fluka, and of the highest purity available.

Acknowledgements

We wish to thank S.Reutener, B.Garcia, J.Brenner and R.Zehnle for expert technical assistance, M.D.Graves, Hoffmann-LaRoche, Nutley, NJ, for the gift of antibodies against protease Peptides I and II, as well as for communication of protease data prior to publication, W.Bannwarth for providing synthetic oligonucleotides, D.Stüber for expression plasmids and M.Brockhaus for the p24 mAb. The logistical support of Drs D.P.Bloxham and A.T.Kröhn, Roche Products Ltd, Welwyn Garden City, UK, is also acknowledged.

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Received on March 21, 1988; revised on April 20, 1988